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Functional association of the stress-responsive LiaH protein and the minimal TatAyCy protein translocase in *Bacillus subtilis*

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ABSTRACT

The bacterial twin-arginine (Tat) pathway serves in the exclusive secretion of folded proteins with bound co-factors. While Tat pathways in Gram-negative bacteria and chloroplast thylakoids consist of conserved TatA, TatB and TatC subunits, the Tat pathways of *Bacillus* species and many other Gram-positive bacteria stand out for their minimalist nature with the core translocase being composed of essential TatA and TatC subunits only. Here we addressed the question whether the minimal TatAyCy translocase of *Bacillus subtilis* recruits additional cellular components that modulate its activity. To this end, TatAyCy was purified by affinity- and size exclusion chromatography, and interacting co-purified proteins were identified by mass spectrometry. This uncovered the cell envelope stress responsive LiaH protein as an accessory subunit of the TatAyCy complex. Importantly, our functional studies show that Tat expression is tightly trailed by LiaH induction, and that LiaH itself determines the capacity and quality of TatAyCy-dependent protein translocation. In contrast, LiaH has no role in high-level protein secretion via the general secretion (Sec) pathway. Altogether, our observations show that protein translocation by the minimal Tat translocase TatAyCy is tightly intertwined with an adequate bacterial response to cell envelope stress. This is consistent with a critical need to maintain cellular homeostasis, especially when the membrane is widely opened to permit passage of large fully-folded proteins via Tat.

1. Introduction

To grow, thrive, and survive, bacteria must direct proteins to their cytoplasmic membrane, cell wall and extracytoplasmic milieu. Accordingly, the Gram-positive bacterium *Bacillus subtilis* has evolved to secrete many different proteins, mostly enzymes, into its natural habitat, the soil and plant rhizosphere [1]. This requires dedicated machinery that drives proteins into and across the membrane. To this end, *B. subtilis* employs two highly conserved pathways for protein transport, namely the general secretion (Sec) pathway and the twin-arginine (Tat) pathway. The Tat pathway stands out by its specialization in the export of fully folded proteins that often contain cofactors [2–6]. These Tat substrates are defined by a consensus S/T-R-R-X-F-L-K motif, including the so-called 'twin-arginine' residues, in the N-terminal signal peptides that predestine them for export from the cytoplasm [7,8].

The Tat machinery of *B. subtilis* entails two translocases that act in parallel. Each translocase is minimal in the sense that it requires only two components termed TatA and TatC for activity [9–11]. The TatA component is relatively small (6–7.4 kDa) and has one N-terminal transmembrane domain [12,13], whereas TatC has six trans-membrane domains (28–28.9 kDa) [11,14–17]. The core translocase TatAyCy is composed of the constitutively expressed TatAy and TatCy proteins. It is known to direct the Rieske iron-sulfur protein QcrA into the cytoplasmic membrane [18], the metallophosphoesterase YkuE to the cell wall [19], and the hemoprotein EfeB both to the membrane-cell wall interface and extracellular milieu [20]. The second translocase TatAdCd, composed of TatAd and TatCd, is detectable only under phosphate starvation conditions where it facilitates secretion of the phosphodiesterase PhoD [9,21]. Of note, minimal Tat translocases as typified by *Bacillus* TatAyCy lack a third component known as TatB, which is common to translocases of Gram-negative bacteria and

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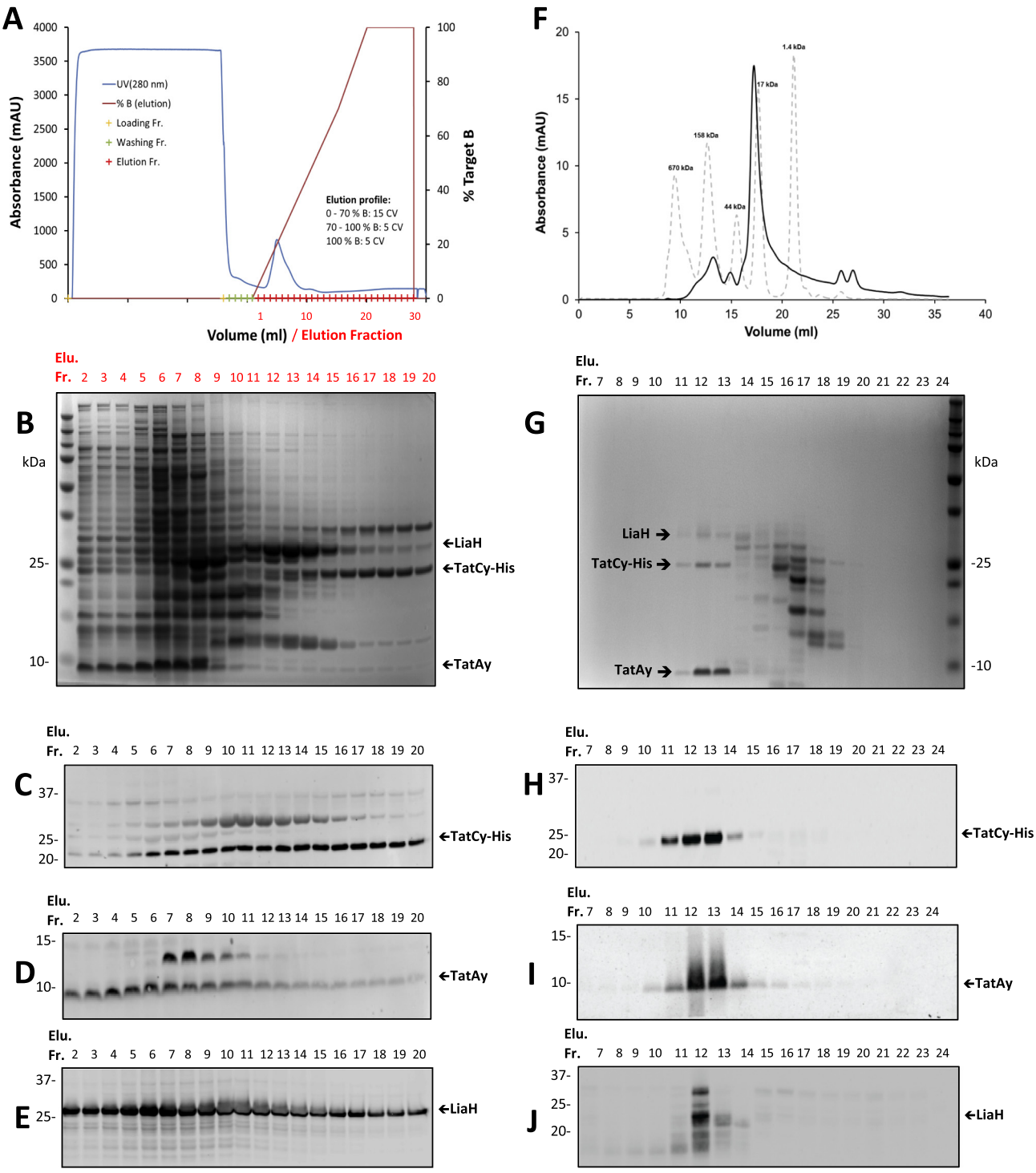

Fig. 1. Co-purification of LiaH with TatAyCy.

(A) The cytoplasmic membrane fraction of *B. subtilis* NZ8900 overexpressing TatAyCy-His was solubilized with 0.1% DDM and subject to metal affinity chromatography. Proteins in elution fractions 2–20 collected during metal affinity chromatography (marked red in A) were separated by LDS-PAGE and visualized by SimplyBlue staining (B) or Western blotting and immunodetection with specific antibodies against the His_6 tag on TatCy (C), TatAy (D), or LiaH (E). Elution fractions 10–13 were pooled and subject to size exclusion chromatography on a Superdex 200 (10/300) column (F). Subsequently, the proteins in elution fractions 7–24 were separated by LDS-PAGE and visualized by SimplyBlue staining (G) or Western blotting and immunodetection with specific antibodies against the His_6 tag on TatCy (H), TatAy (I), or LiaH (J). Proteins in panels B and G were identified by MS, and the respective gels with marked protein identifications are shown in Fig. S1 and S2. Note that TatCy runs at ~23 kDa and TatAy at ~9 kDa, which differs from the predicted molecular weight (Mw) of 28.9 and 6 kDa, respectively. LiaH runs at the predicted Mw of ~27 kDa.

thylakoids [22,23]. The role of TatB is performed by bifunctional TatA proteins, as demonstrated for TatAy and TatAd of *B. subtilis* [22,24–26]. Interestingly, *B. subtilis* contains a third TatA protein, TatAc, which supports protein translocation via TatAyCy, but cannot functionally replace TatAy [27].

Since the mechanism of Tat translocation appears to be conserved across species, it has been proposed that, in minimal Tat translocases, a complex of TatA and TatC serves in the docking of cargo proteins [6,16,28]. Subsequently, the docking complex with its bound cargo recruits TatA oligomers in the membrane [6,29,30], leading to the actual translocation of cargo and cleavage of the signal peptide by signal peptidase [21,31]. An intriguing question is whether the minimal Tat translocases of *Bacillus* operate in isolation, or whether they also involve other factors. This question is relevant, as it was previously shown in *Escherichia coli* that the phage shock protein A (PspA) can be detected in association with TatA [32,33]. PspA is a homologue of the IM30 (VIPP1) protein of thylakoid-harboring photosynthetic organisms [34], and it has two paralogues, LiaH and PspA, in *B. subtilis* [35]. Under stress conditions that compromise membrane integrity, the PspA, IM30, and LiaH proteins form high-order oligomeric structures that bind peripherally to the affected membrane regions to mitigate the potentially lethal defects of phospholipid bilayer perturbations [34,36]. Previous studies have furthermore shown that LiaH is up-regulated via the LiaRS two-component regulatory system in response to stresses caused by antibiotics that affect the undecaprenol cycle [37], and by secretion of some heterologous proteins via the Sec pathway [38]. In contrast, PspA up-regulation in *B. subtilis* depends on the alternative sigma factor SigW, which is responsive to cell envelope perturbations and alkaline shock [39–41].

The present study was aimed at identifying potentially new members of the minimal core translocase TatAyCy by using a biochemical approach. Here we show that TatAyCy is less minimal than previously believed, because the stress-responsive LiaH protein is functionally associated with this translocase.

2. Results

2.1. LiaH binds both TatAy and TatCy

To obtain a biochemical perspective on possible interaction partners of the TatAyCy translocase, the TatAyCy complex was overexpressed with a C-terminal hexahistidine tag (His_6) in a *B. subtilis tatAyCy* mutant strain [30]. Importantly, previous studies showed that the His_6 tag does not interfere with TatAyCy translocation activity [30]. To achieve TatAyCy overexpression, the subtilin-inducible SURE system was applied [42]. TatAyCy-overexpressing cells were disrupted by bead-beating, membranes were purified from the disrupted cells, and membrane proteins solubilized in 0.1% Lauryl-β-D-maltoside (DDM) were used for metal affinity chromatography as previously described [30]. Upon extensive washing, bound proteins were eluted from the column with an imidazole gradient, and the different elution fractions were collected. As shown by monitoring the absorption at 280 nm, proteins eluted in one major peak (Fig. 1A). Proteins in the respective elution fractions were separated by lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) and stained with SimplyBlue SafeStain (Fig. 1B). Since multiple proteins eluted, the dominant bands were analyzed by Mass Spectrometry (MS), demonstrating the presence of TatCy-His and TatAy (Fig. S1, Table S1). In addition, the MS analysis revealed the presence of LiaH and the ribosomal proteins RplM, RplR, RpsC, RpsE, RpsJ, RpsS and RplB (Fig. S1). Of note, despite its similarity to LiaH, the PspA protein of *B. subtilis* was not found to co-elute with TatAyCy. To verify the MS identifications, the elution fractions were analyzed by Western blotting using antibodies specific for the His_6 tag on TatCy (Fig. 1C), TatAy (Fig. 1D), and LiaH (Fig. 1E). This revealed that, with an increasing imidazole concentration, increasing amounts of TatCy were co-eluted with decreasing amounts of TatAy, reaching a constant TatAy:TatCy ratio from elution fraction 13 onwards (Fig. 1C,D). Most notably, LiaH was found to be abundantly present in all elution fractions, even at high imidazole concentrations (Fig. 1E), showing an elution profile similar to that of TatAy (Fig. 1D,E). To assess the specificity of the observed co-isolation of LiaH with TatAyCy-His, metal affinity chromatography was performed under the same conditions using lysates of two control strains that did not overexpress TatAyCy (i.e. *B. subtilis* NZ8900 and *B. subtilis* NZ8900 carrying the empty vector pNZ8910). In these experiments, no TatAy or TatCy-His was eluted from the column (as expected) but, importantly, the detectable amounts of LiaH were also negligible compared to the amounts of LiaH co-eluted with TatAyCy in the TatAyCy-overexpressing strain (data not shown). Together, these findings were suggestive of possible interactions between LiaH and the eluted TatAyCy complexes, interactions between TatAy and LiaH in particular.

To verify a possible TatAyCy-LiaH interaction, elution fractions 10–13 were pooled, concentrated and subjected to size exclusion chromatography. The respective chromatograms showed three major peaks with masses of ~150–600 kDa (fractions 11–13), ~100 kDa (fraction 14–15) and ~20 kDa (fractions 16–18; Fig. 1F). Next, the collected fractions were subjected to LDS-PAGE and the separated proteins were visualized by SimplyBlue staining. This revealed that fractions 11–13, corresponding to the elution peak of ~150–600 kDa, encompassed three dominant protein bands that MS identified as TatAy, TatCy and LiaH (Fig. 1G, Fig. S2). The other peak fractions were mostly composed of ribosomal proteins, including RplB, RplC, RplM, RplR, RpsB, RpsC, RpsD, RpsJ and RpsS (Fig. S2). Western blotting showed that the 150–600 kDa peak includes complexes of TatAyCy and LiaH (Fig. 1, H-J; Fig. S2), which validated the MS data. Taken together, these results suggested close interactions between the overexpressed minimal TatAyCy translocase and the LiaH protein of *B. subtilis*.

Since LiaH is known to be upregulated upon stress in the membrane and cell wall [37,43], we decided to further explore its interactions with TatAyCy by co-immunoprecipitation (co-IP) experiments. For this purpose, membranes of TatAyCy-His-overexpressing cells of the wild-type *B. subtilis* SURE strain NZ8900 were solubilized and incubated with protein A dynabeads that had been pre-incubated with anti-His_6 antibodies. Next, the beads were washed and bound proteins were eluted with 2× LDS loading buffer and subjected to LDS-PAGE and Western blotting. As shown in Fig. 2A-C, along with TatCy-His, the TatAy and LiaH proteins co-eluted from the beads, showing that LiaH directly interacts with TatAyCy. The next question we asked was whether LiaH interacts with TatAy, TatCy or both. To this end, TatAy-His or TatCy-His were individually overexpressed in *B. subtilis* using the SURE system and co-IP was performed on solubilized membrane proteins from the respective overexpressing strains. Of note, we were unable to overproduce TatAy-His in the wild-type NZ8900 strain, whereas this was possible in the genome-reduced *B. subtilis* strain IIG-Bs27–47-24. Fig. 2D,E shows that indeed, LiaH eluted from the beads along with the overexpressed TatAy-His. Unexpectedly however, LiaH also co-eluted from the beads with the overexpressed TatCy-His (Fig. 2F,G). Since the co-IP of LiaH with TatCy-His was performed in a TatAy-proficient background, we wanted to know whether this reflected a direct interaction of TatCy and LiaH, or an indirect interaction via the chromosomally-encoded TatAy. To this end, we repeated the co-IP experiment with TatCy-His overexpressed in a *tatAyCy*-deficient strain. Also, in the absence of TatAy, LiaH was found to co-immunoprecipitate with TatCy-His, indicating a direct interaction between TatCy-His and LiaH (Fig. S3A–C). As a negative control, we also subjected membranes of non-overexpressing *B. subtilis* NZ8900, grown in the presence of 1% subtilin, to this same procedure. As expected, neither TatCy-His nor LiaH were detectable in the elution fraction (Fig. S3D,E). Further, we wondered whether the His_6 tag on TatCy might have an effect on the co-IP of LiaH with overexpressed TatAyCy. Therefore, we performed the same experiment using a strain that overexpresses TatAyCy with a StrepII tag attached to TatCy (Fig. S4). Also when TatCy contained the StrepII tag,

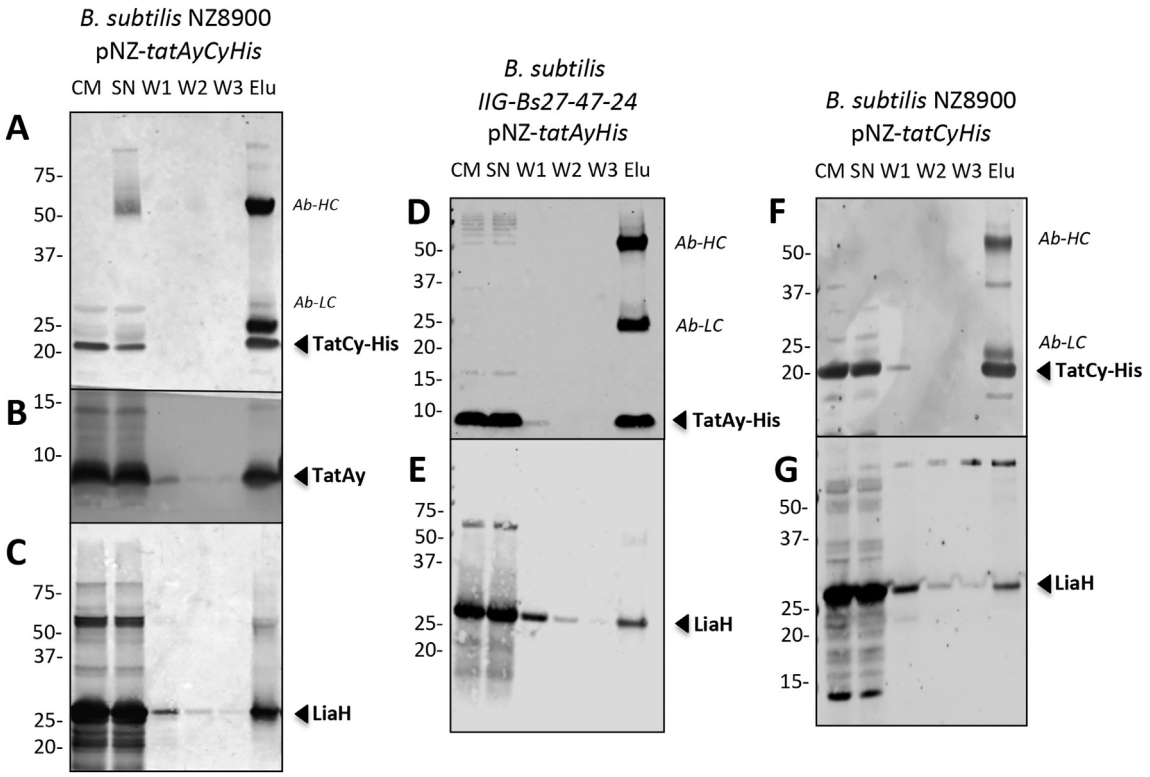

Fig. 2. Co-immunoprecipitation of LiaH with TatAyCy, TatCy, and TatAy.
Solubilized cytoplasmic membranes (CM) from *B. subtilis* NZ8900 overexpressing TatAyCy-His (A-C), *B. subtilis* IIG-Bs27–47-24 overexpressing TatAy-His (D, E), or *B. subtilis* NZ8900 overexpressing TatCy-His (F, G) were incubated for 1 h with His_6-specific antibodies bound to Protein A dynabeads. Subsequently, the beads were separated from the sample with a magnet. The supernatant (SN) was collected, the beads were washed three times (W1–3), and proteins were eluted (Elu) with LDS loading dye. Proteins in the different fractions were separated by LDS-PAGE and analyzed by Western blotting with specific antibodies for the His_6 tag (A, D, F), TatAy (B), or LiaH (C, E, G). Ab-HC/LC, antibody heavy/light chain. Negative control co-IP experiments performed on solubilized cytoplasmic membranes from the non-Tat overexpressing wild-type strain 168 and a TatAc-overexpressing strain are respectively shown in Figs. S3 and 6.

we observed co-IP of TatAy and LiaH with TatCy (Fig. S4A–C). Moreover, using antibodies specific for TatAy in the same experimental setup, TatCy-Strep and LiaH were co-immunoprecipitated with TatAy (Fig. S4D–F). Conversely, using antibodies specific for LiaH, TatCy-Strep and TatAy were co-immunoprecipitated with LiaH (Fig. S4G–I). Altogether, these 'post-mortem' analyses show that LiaH can tightly bind both TatAy and TatCy, even if the two Tat proteins are expressed in isolation.

2.2. TatAyCy expression and LiaH induction are intimately linked in vivo

LiaH is a known member of the LiaRS regulon, which senses and responds to perturbations in the membrane and cell wall milieu as imposed by antimicrobials, such as bacitracin [37,44]. Since it has been proposed that Tat-dependent protein translocation could rely on membrane-weakening [5,45,46], we sought to identify a possible relationship between TatAyCy expression and LiaRS activation in vivo, which would lead to increased LiaH levels. As a first approach, the expression levels of LiaH in response to TatAyCy overexpression were measured by Western blotting (Fig. 3A). Indeed, titration of TatAyCy expression with increasing amounts of subtilin resulted in concomitantly increasing LiaH levels with an optimum at 5% subtilin. In contrast, LiaH was barely detectable in the TatAyCy-proficient wild-type control strain, irrespective of the amounts of subtilin added (Fig. 3A). These results indicate that the two-component regulatory system LiaRS senses TatAyCy overexpression. Similarly, the LiaH levels increased strongly when TatAy or TatCy were overexpressed individually (Fig. S5). Here it should be noted that TatAy expression in the IIG-Bs27–47-24 strain was already saturated in absence of the subtilin inducer leading to high-level induction of LiaH expression, whereas LiaH expression followed the titratable TatCy-His expression in the NZ8900 strain.

In the presence of cell envelope stress, the *liaI* promoter (P_{liaI}) will be activated leading to transcription of the downstream *lia* operon, which includes *liaH* [37]. Activation of the LiaRS system can thus be measured with a previously constructed P_{liaI} *lux* reporter cassette, where promoter activity leads to expression of luciferase that can be quantified by recording light emission [47]. To validate the Western blotting data in Fig. 3A, we assessed whether and to what extent TatAyCy overexpression would cause P_{liaI} *lux* activation-dependent light emission. As shown in Fig. 3E,F, subtilin-induced expression of TatAyCy led to high-level P_{liaI} activation. Of note, due to some leakiness of the SURE expression system, low-level activity of P_{liaI} was also detectable in the absence of subtilin (Fig. 3E), but in terms of light emission this represented < 1% of the P_{liaI} activity measured when TatAyCy expression was fully induced (Fig. 3F; please note the different y-axes scales in panels E and F). On the other hand, exposure of the non-Tat-overexpressing strain carrying the P_{liaI} *lux* reporter to 1% subtilin did not result in any detectable light emission (Fig. 3F). Unexpectedly, the P_{liaI} activity recorded in the presence of 30 μg/ml bacitracin, a known inducing cue for P_{liaI} [48], reached maximally ~20% of the induction level that was reached upon fully induced TatAyCy expression (Fig. 3G). These observations, together with the Western blotting data in Fig. 3A-C, imply that the LiaRS system is super-responsive to the expression of TatAyCy. In fact, compared to the previously described inducer bacitracin, TatAyCy expression is a much stronger stimulus for the LiaRS system.

Since the cellular LiaH level was increased upon TatAyCy overexpression, we asked the question whether the LiaH level might be reduced upon *tatAyCy* deletion. However, this was not the case, as

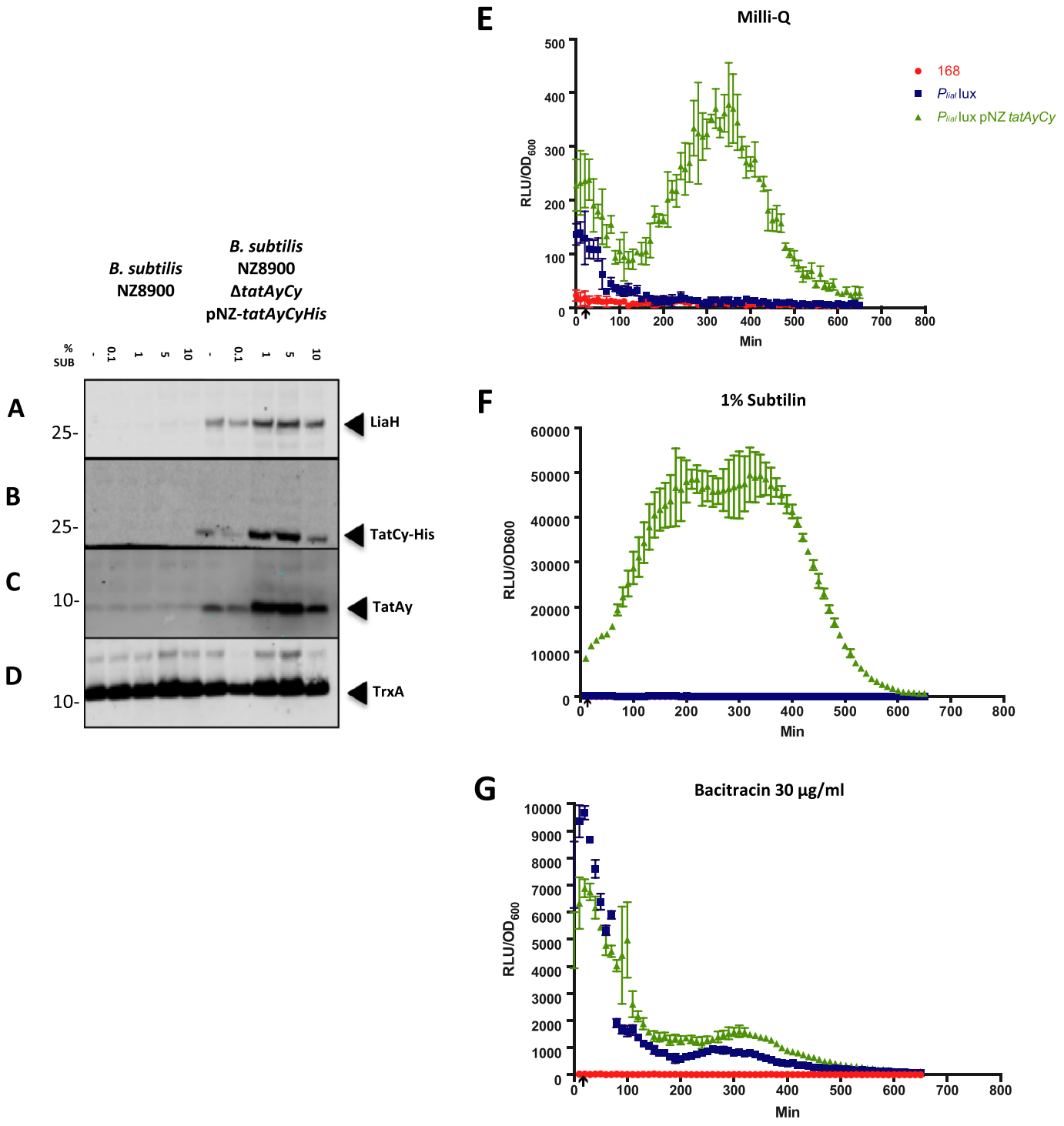

Fig. 3. TatAyCy overexpression is trailed by LiaH induction.
Cells of *B. subtilis* NZ8900 or *B. subtilis* NZ8900 Δ*tatAyCy* pNZ-*tatAyCyHis* were exposed for 5 min to varying concentrations of subtilin (0–10%) to induce TatAyCy-His overexpression. Culture samples were collected and normalized according to the respective OD_{600}. Subsequently, cells were pelleted by centrifugation, and disrupted by bead-beating. Proteins from the disrupted cells were separated by LDS-PAGE and analyzed by Western blotting with antibodies specific for LiaH (A), the His_6 tag on TatCy (B), TatAy (C), or the cytoplasmic control protein TrxA (D). To assess P_{liaI} induction, exponentially growing cells of *B. subtilis* 168, *B. subtilis* 168 P_{liaI}-*lux*, or *B. subtilis* NZ8900 P_{liaI}-*lux* pNZ-*tatAyCyHis* were diluted in fresh LB medium to an OD_{600} of ~0.015 and aliquots of 150 µL were transferred to a black-bottomed 96-well plate and incubated. Subtilin (1%), Bacitracin (30 µg/ml), or Milli-Q water (1%) were added when the cells had reached an OD_{600} of ~0.1 (marked with an arrow). The relative light units (RLU) related to *lux* gene expression and the OD_{600} were measured over time. (E) Luciferase activity produced by the investigated P_{liaI}-*lux* reporter strains in the absence of inducing cues (1% Milli-Q water was added as a negative control), (F) upon TatAyCy-His overexpression induced by 1% subtilin, or (G) upon LiaRS activation with 30 µg/mL bacitracin. Upward pointing arrows mark the time point at which milli-Q, subtilin or bacitracin were added.

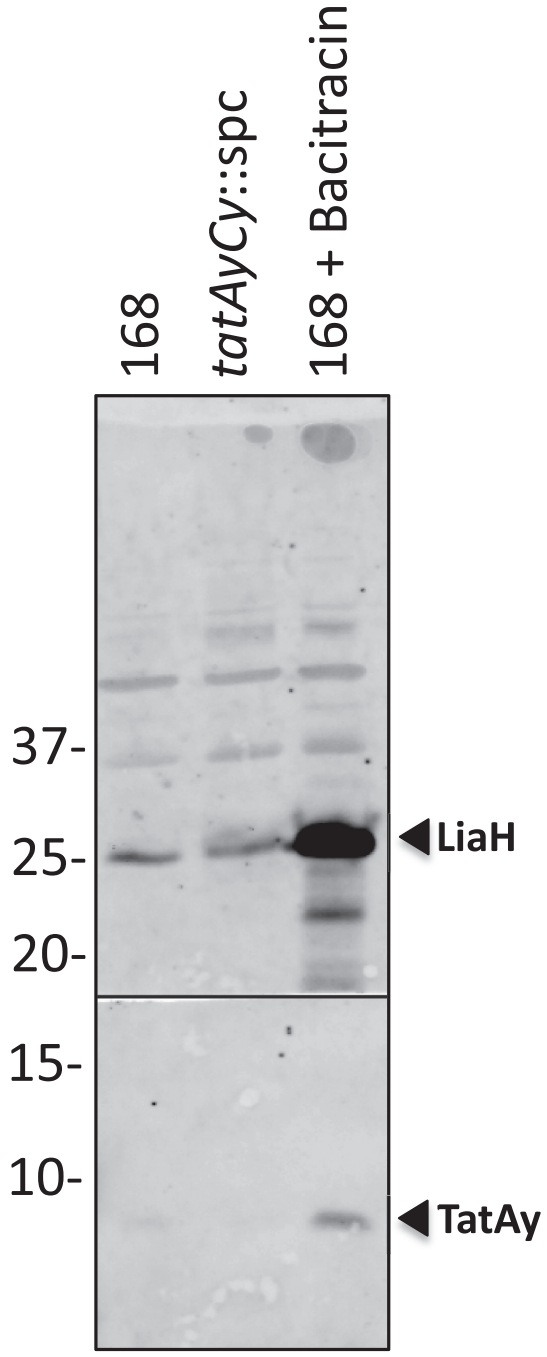

Fig. 4. Bacitracin elicits an elevated cellular level of TatAy.
The expression levels of LiaH were assessed by Western blotting using wild-type *B. subtilis* 168 and a *tatAyCy*-deficient *B. subtilis* 168 mutant strain. Cells were grown to an OD_{600} of ~3 in LB supplemented with 1% xylose. As a control for LiaH induction, *B. subtilis* 168 was also grown in the presence of 30 μg/ml bacitracin. Culture samples were collected and normalized according to the respective OD_{600}. Subsequently, bacteria were collected by centrifugation, disrupted by bead-beating, and subjected to LDS-PAGE, Western blotting, and immunodetection with specific antibodies against TatAy or LiaH.

shown by Western blotting (Fig. 4), which is in line with the fact that the native expression levels of TatAy and TatCy are very low [9,21,49]. Remarkably, we noticed instead that the cellular level of chromosomally-encoded TatAy was significantly increased when LiaH overproduction was provoked with bacitracin (Fig. 4). Since *tatAy* expression is neither controlled by LiaRS nor by SigW [40,41,50], this observation suggests that TatAy is stabilized by overproduction of LiaH in the presence of bacitracin. This would be consistent with the observed interaction between TatAy and LiaH.

2.3. Overexpression of the Tat substrate EfeB or the third TatA protein of B. subtilis, TatAc, does not have a significant impact on LiaH levels

To determine whether the induction of LiaH is specific for TatAyCy overexpression, or whether other Tat-related proteins might also lead to LiaH induction, we investigated the levels of LiaH upon induced overexpression of the TatAyCy substrate EfeB using the SURE system. Of note, EfeB is a DyP-type peroxidase that oxidizes ferrous iron in a first step of iron acquisition via the membrane-embedded EfeUOB system [20]. Accordingly, most EfeB is found in a membrane-associated form. As shown in Fig. 5, induction with subtilin for 5 min or 2 h resulted in overexpression of EfeB, which was associated with low-level induction of LiaH. However, this LiaH induction remained marginal compared to the massive levels of LiaH that were produced upon TatAyCy overproduction from the same promoter under the same experimental conditions (Fig. 5). This implies that the strong LiaH response is highly specific for the TatAyCy translocase, rather than for its substrate EfeB. Of note, the overexpression of neither TatAyCy nor EfeB led to detectable release of the cytoplasmic marker protein thioredoxin A (TrxA) into the growth medium, which indicates that the integrity of the cells was not affected under these conditions [51].

For a fair comparison of the LiaRS-mediated response to TatAyCy overexpression, we also wanted to know whether a similar effect could be observed in response to the overexpression of a related membrane protein. To this end, we overexpressed the TatAc protein using the SURE system. Importantly, TatAc is structurally similar to TatAy and it can aid in the function of TatAyCy [27]. As shown in Fig. 6A-C, the overexpression of TatAc resulted in only a very minor raise in the cellular LiaH level compared to the overexpression of TatAyCy, or compared to overexpression of TatAy or TatCy alone (Fig. S5). Instead, the observed induction of LiaH was comparable to the level observed upon EfeB overexpression (Fig. 5). The observation that TatAc did not lead to severe overexpression of LiaH was further exploited to serve as an additional negative control for the co-IP experiments presented in Figs. 2 and S3. Indeed, as in the latter experiments, co-IP of TatAy, TatAc or LiaH from TatAc-overproducing cells was not observed with the anti-His_6 antibodies (Fig. 6D-G). Together, these observations show that the massive induction of LiaH upon TatAyCy overproduction is specific for the TatAyCy translocase.

2.4. LiaH determines the quantity and quality of TatAyCy activity

Having observed a direct interation of LiaH with TatAyCy, as well as super-induction of LiaH expression by TatAyCy overexpression, we wanted to know whether the presence or absence of LiaH makes a difference for TatAyCy activity. To this end, we first examined the secretion of EfeB, which was expressed from a xylose-inducible promoter (X-*efeB*) as the endogenous cellular and secreted levels of EfeB are relatively low (Fig. 7A,B). Further, we compared the physiological levels of TatAyCy production to a situation where TatAyCy was overexpressed about 5-fold from the low-copy number plasmid p*CAyCy* [21,52]. The levels of EfeB in cell and growth medium fractions were then monitored by Western blotting. The results in Fig. 7A,B show that the overall level of EfeB in cells and medium of the wild-type strain 168 were substantially enhanced upon xylose induction of EfeB expression, while TatAyCy overexpression by itself had no effect. Combined overexpression of EfeB and TatAyCy in the otherwise wild-type 168 background did, however, lead to a significantly enhanced level of EfeB secretion (Fig. S6A). As expected, an increased level of LiaH in the cells was observed upon TatAyCy overexpression (Fig. 7C). This implies that higher amounts of the TatAyCy translocase and possibly LiaH allow for enhanced secretion of the EfeB substrate. Very different results were obtained in a *liaH* mutant background. In particular, the absence of

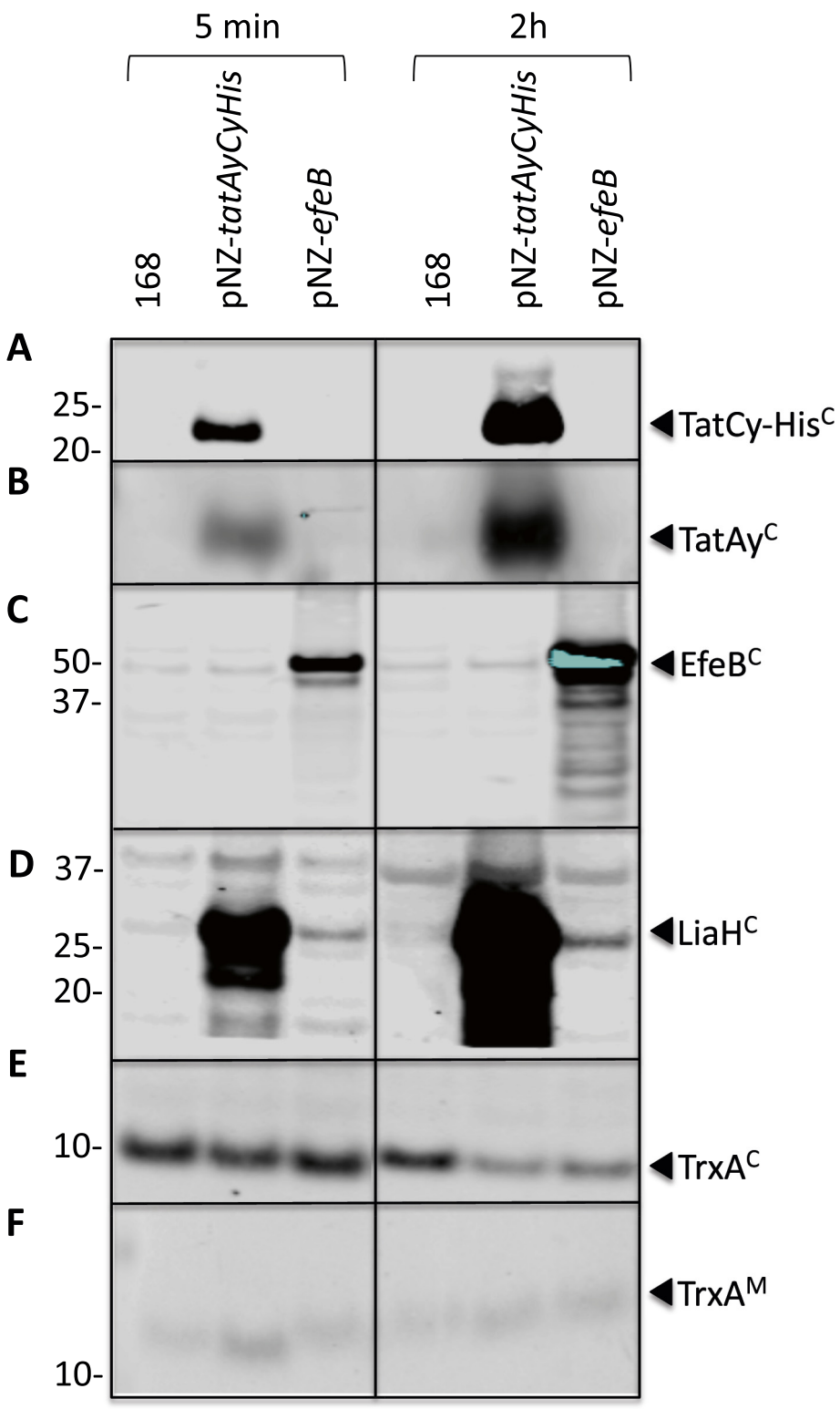

Fig. 5. EfeB overexpression elicits only marginal LiaH induction.

Expression levels of LiaH were compared upon overexpression of TatAyCy or the TatAyCy substrate EfeB in *B. subtilis* NZ8900, and wild-type *B. subtilis* 168 was used as a control. Cells were grown to an OD_{600} of ~0.8 and induced with 1% subtilin for 5 min or 2 h. Culture samples were collected and normalized according to the respective OD_{600}. Proteins in the cell (labelled ^{C}) and medium fractions (labelled ^{M}) were separated by centrifugation and subjected to LDS-PAGE, Western blotting, and immunodetection with specific antibodies against the His_6 tag on TatCy (A), TatAy (B), EfeB (C), LiaH (D), or the cytoplasmic marker protein TrxA (E,F) as indicated. Note that the growth medium fractions were 3-fold concentrated compared to the cell fractions.

LiaH led to a substantial reduction of EfeB secretion in the strain overexpressing both EfeB and TatAyCy (Figs. 7A,B and S6A). This means that LiaH is indeed needed for optimal EfeB secretion via TatAyCy.

Intriguingly, the combined overexpression of TatAyCy and its EfeB substrate led to increased release of LiaH into the growth medium (Fig. 7D), suggesting an increase in membrane permeability for LiaH under these conditions. As shown with the cytoplasmic marker protein TrxA, this cannot be related to unspecific protein leakage into the growth medium as no changes in extracellular TrxA were detectable, irrespective of the presence or absence of LiaH (Fig. 7E,F). This implies that, under the tested conditions, membrane integrity was not affected by LiaH-deficiency, and that effects of the absence of LiaH on EfeB secretion must be attributed to its interaction with TatAyCy. Likewise, the increased release of LiaH into the growth medium upon induced expression of TatAyCy and its EfeB substrate is apparently not

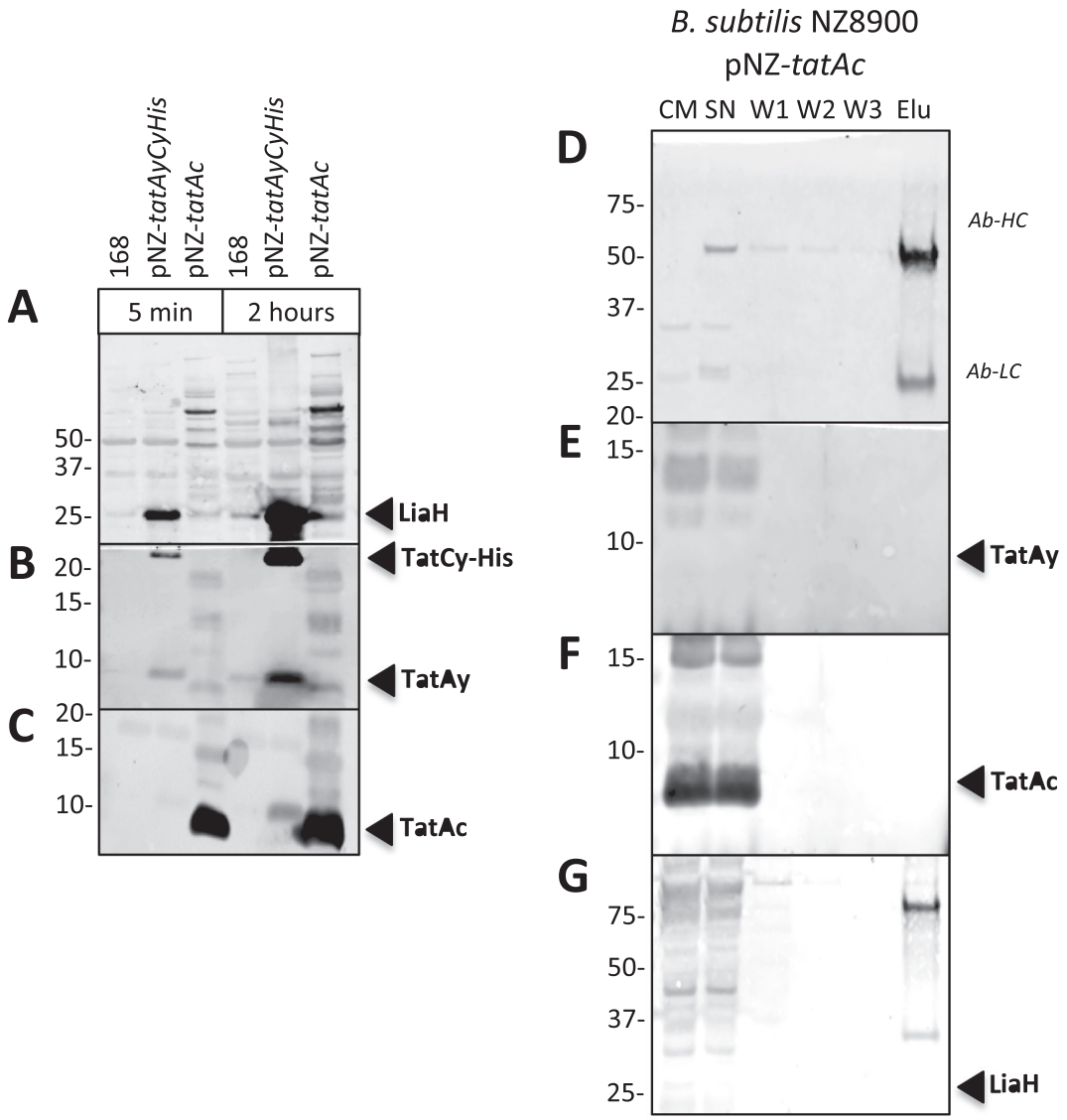

Fig. 6. TatAc overexpression does not lead to LiaH induction.
Expression of TatAyCy-His or TatAc in *B. subtilis* NZ8900 was induced with 1% subtilin, and the wild-type strain 168 was used as a control. At 5 min or 2 h post induction with subtilin, culture samples were collected and normalized according to the respective OD_{600}. Cells were separated from the medium by centrifugation, and the cellular proteins were analyzed by LDS-PAGE and Western blotting with specific antibodies against LiaH (A), the His_6 tag on TatCy (B), TatAy (B) or TatAc (C). Subsequently, a control co-IP experiment was performed as in Figs. 2 and S3, where cytoplasmic membranes (CM) from *B. subtilis* NZ8900 overexpressing TatAc were isolated, solubilized and incubated for 1 h with His_6-specific antibodies bound to Protein A dynabeads. The beads were then separated from the sample with a magnet. The supernatant (SN) was collected, the beads were washed three times (W1–3), and proteins were eluted (Elu) with LDS loading dye. Proteins in the different fractions were separated by LDS-PAGE and analyzed by Western blotting with specific antibodies for the His_6 tag (D), TatAy (E), TatAc (F), or LiaH (G). Ab-HC/LC, antibody heavy/light chain.

unspecific but, instead, it is seemingly related to the intimate interaction of LiaH with the translocase. This interaction may set a limit to the level of EfeB secretion.

Of note, as shown in Fig. 7A,B, TatAyCy does have activity in the absence of LiaH, indicating that LiaH is an accessory subunit to TatAyCy rather than an essential component. This is fully consistent with previous observations showing that TatAyCy heterologously expressed in *E. coli* is active, albeit that it cannot fully replace the native *E. coli* TatABC translocase [23,53,54].

The Rieske iron-sulfur protein QcrA is an abundantly produced menaquinol:cytochrome *c* oxidoreductase in the membrane-embedded cytochrome *bc*1 complex of *B. subtilis*, which requires TatAyCy activity for correct membrane insertion [18,55]. Importantly, we have previously shown that a fraction of membrane-assembled QcrA (denoted QcrA*) is aberrantly cleaved by signal peptidase and secreted in a TatAyCy-dependent manner [18,55]. The secreted QcrA fraction can thus be used as a read-out for TatAyCy activity [18,55]. Therefore, we also inspected the impact of a *liaH* deletion on QcrA secretion, using cells that allow for individual or combined overexpression of EfeB and TatAyCy. Firstly, as shown in Fig. 7G,H, overexpression of EfeB alone or in combination with TatAyCy had relatively moderate effects on the cellular levels of QcrA in the wild-type and *liaH* mutant strain. Further, the xylose-induced expression of EfeB [9,21] led to a severe reduction in the secretion of QcrA* in the wild-type strain, indicating that EfeB competes with QcrA for export via TatAyCy (Fig. S6B). Intriguingly, this competition cannot be relieved by TatAyCy overexpression alone, even in the wild-type background where the LiaH level is increased (Fig. 7I) due to the plasmid-mediated TatAyCy overexpression (Fig. 7J). This suggests that another, as yet, unidentified factor is insufficiently available for QcrA export under these conditions. Importantly however,

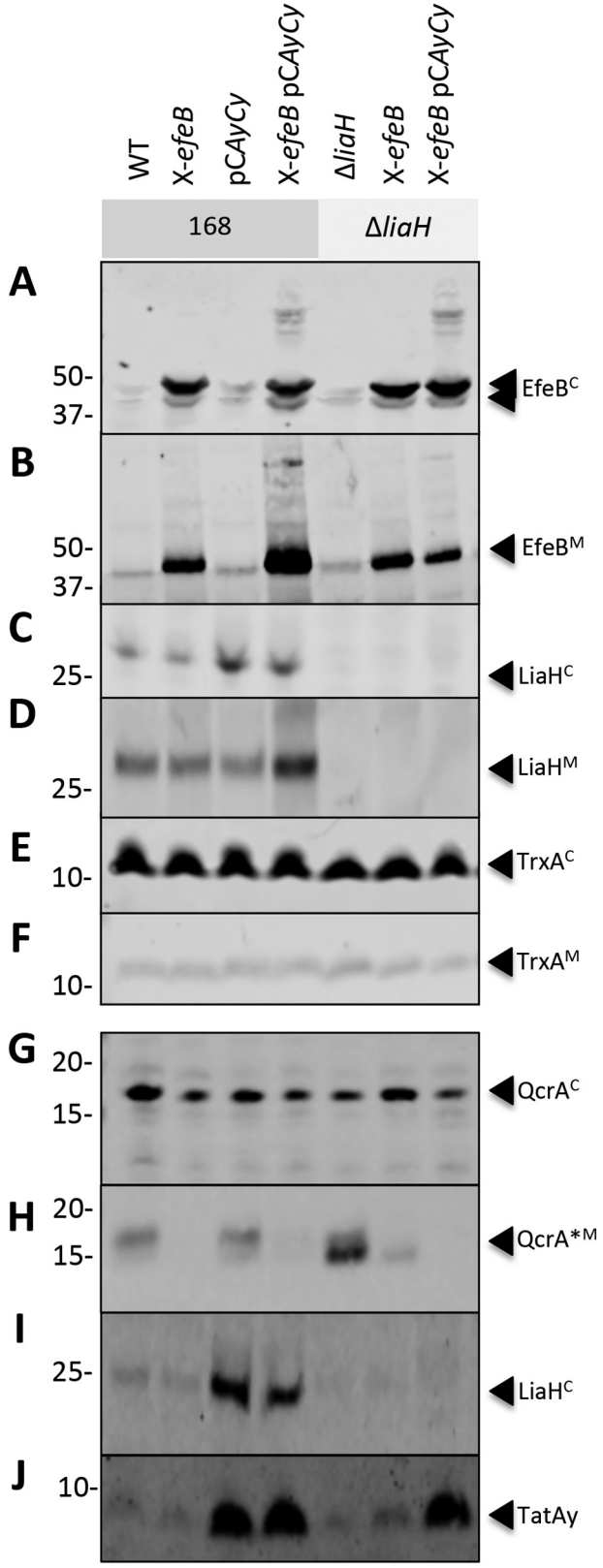

Fig. 7. LiaH determines the quantity of EfeB secretion and the quality of QcrA export.
EfeB overexpression in the wild-type *B. subtilis* 168 background, or a *liaH*-deficient mutant (Δ*liaH*) was induced with 0.5% xylose (A-F) or 1% xylose (G-J) using a xylose-inducible promoter fused to *efeB*-myc (X-*efeB*). Constitutive TatAyCy overexpression was achieved using plasmid pC*AyCy*. Culture samples were collected and normalized according to the respective OD_{600}. Proteins in the growth medium (labelled ^{M}) and cell fractions (labelled ^{C}) were separated by centrifugation and subjected to LDS-PAGE, Western blotting, and immunodetection with specific antibodies against EfeB (A, B), LiaH (C, D, I), the cytoplasmic marker TrxA (E, F), QcrA (G, H), or TatAy (J). Note that the growth medium fractions were 3-fold concentrated compared to the cell fractions. A quantification of the secreted levels of EfeB and QcrA* is shown in Fig. S6.

compared to the wild-type situation, the level of a smaller form of QcrA* is drastically increased if LiaH is absent (Fig. S6B). This shows that LiaH sets a limit to the aberrant signal peptidase cleavage of QcrA and subsequent secretion of the processed QcrA* form into the growth medium. The high-level QcrA* secretion in the *liaH* mutant is reduced upon EfeB expression, in line with the apparent competition of these TatAyCy substrates in the wild-type background, and it is non-detectable if TatAyCy is co-overexpressed with EfeB (Figs. 7G,H and S6B). Altogether, these observations demonstrate a role for LiaH in the quality of TatAyCy-mediated QcrA assembly in the membrane.

2.5. Absence of LiaH does not affect high-level Sec-dependent secretion of AmyE

Previous studies have shown that LiaH can be upregulated in response to high-level secretion of the heterologous α-amylase AmyQ via the Sec pathway of *B. subtilis*, suggesting a possible relationship between LiaH and Sec-dependent protein secretion [38]. Also, it was conceivable that a deletion of *liaH* could cause defects in the cytoplasmic membrane that would indirectly result in altered protein secretion via the Tat pathway. Therefore, we investigated whether Sec-dependent protein secretion might be affected by a *liaH* mutation. To this end, we overexpressed the *B. subtilis* α-amylase AmyE to semi-industrial levels (g/l) in a *liaH* mutant strain, or the respective wild-type strain, and compared the resulting secretion levels of AmyE by LDS-PAGE. As shown in Fig. 8, the absence of LiaH did not detectably affect the high-level secretion of AmyE. This shows that Sec-dependent protein secretion by *B. subtilis* is not affected in the absence of LiaH. In turn, this implies that the here-described effects of the *liaH* mutation on Tat-dependent protein secretion in *B. subtilis* cannot be attributed to aspecific effects on membrane integrity, membrane polarization or the energy status of *liaH* mutant cells that would be expected to affect both Sec- and Tat-dependent protein secretion [56,57].

3. Discussion

The present study was aimed at identifying possible partner proteins of the *B. subtilis* TatAyCy preprotein translocase through a biochemical approach. This led to the identification of the cell envelope stress-responsive LiaH protein as an accessory subunit of TatAyCy. The association between TatAyCy and LiaH was sufficiently strong to allow co-purification of LiaH with TatAyCy-His upon metal affinity chromatography and subsequent gel filtration, or via co-IP experiments with antibodies specific for a His_6 tag or a StrepII tag on TatCy, and co-IP with antibodies specific TatAy or LiaH.

As evidenced by gel filtration, the detected TatAyCy-LiaH complexes ranged in size between ~150–600 kDa. This substantial variation in size is indicative of complexes containing variable amounts of TatAy and/or LiaH, as is also suggested by the elution profiles observed for TatCy-His, TatAy and LiaH upon metal affinity chromatography. Here the elution profiles of TatAy and LiaH resembled each other, whereas the elution profile of TatCy-His was different. This observation on the TatAyCy complex of *B. subtilis* is fully in line with the results from a recent study in *E. coli*, where it was shown that three major, differently-sized, TatABC complexes can form, depending on the amount of TatA that is bound [58]. For the here identified *B. subtilis* TatAyCy-LiaH complexes, it is conceivable that the variable complex

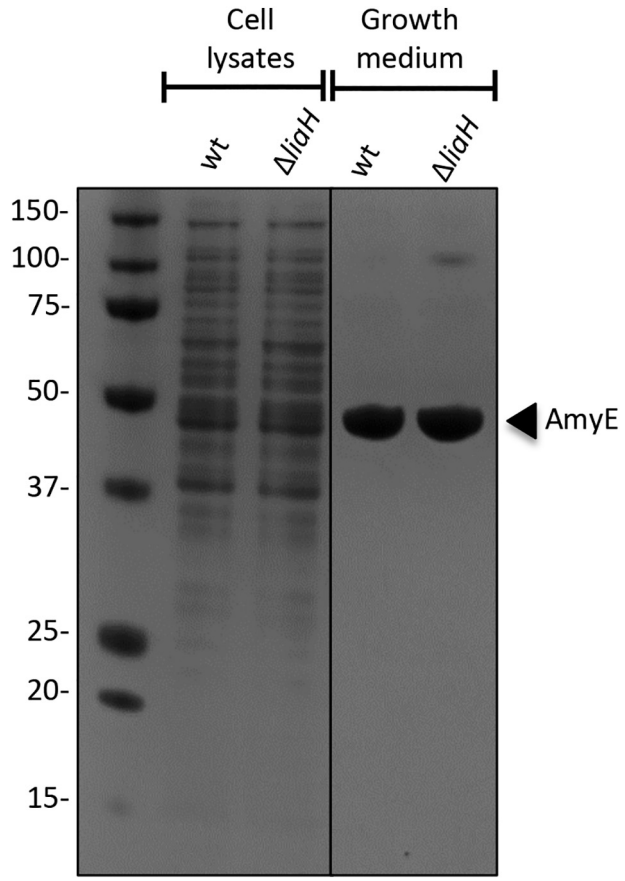

Fig. 8. Absence of LiaH does not affect high-level secretion of AmyE via Sec. Wild-type (wt) or *liaH* mutant (Δ*liaH*) cells of *B. subtilis* strain CB15–14 overproducing the *B. subtilis* α-amylase AmyE were grown for 16 h in MBU medium. Culture samples were collected and normalized according to the respective OD_{600}. Subsequently, cells were separated from the growth medium by centrifugation. Proteins in the cell and growth medium fractions were separated by LDS-PAGE and visualized by SimplyBlue staining.

size may not only relate to varying amounts of TatAy, but also to varying amounts of LiaH. If so, the smallest complexes observed by size exclusion chromatography would have relatively little LiaH and TatAy bound, as is the case for the complexes that eluted from the metal affinity chromatography resin at the highest imidazole concentrations. Conversely, complexes with a relatively high LiaH load will be larger, also because LiaH has the intrinsic propensity to form high-order oligomeric structures [36,59]. Of note, next to the possibility that the observed complexes have recruited differing amounts of TatAy and LiaH, it is also conceivable that TatAy is dissociating to some extent from TatAyCy complexes during metal affinity- and size exclusion chromatography, along with a fraction of the bound LiaH. At present, we cannot distinguish between these possible scenarios.

A novel finding was that LiaH interacts both with TatAy and TatCy. Especially the latter finding was unexpected as the elution profile of LiaH upon metal affinity chromatography of TatAyCy-His was more similar to the elution profile of TatAy than that of TatCy-His. Previous studies have shown that PspA of *E. coli* and VIPP1 of *Synechocystis* sense areas in the membrane with high 'stored curvature elastic stress' (SCE stress) and packing defects. Both proteins are thought to help in stabilizing the membrane by binding to areas with high SCE stress in the form of high-order oligomeric structures [60,61]. In *E. coli,* overexpression of TatA has been shown to lead to activation of the Psp response due to membrane destabilization caused by high levels of TatA, which in turn could lead to SCE stress [46,62,63]. In addition, high levels of TatA led to changes in the transmembrane proton gradient (ΔpH). PspA seems to counteract this effect by interacting with TatA [62,63]. It has been proposed that LiaH of *B. subtilis* supports membrane stabilization in a similar manner in response to membrane stress-inducing cues [34,57]. In particular, Dominguez et al. showed that membrane stabilization by LiaH requires the interaction with its membrane anchor, LiaI, in static spots [34,36]. Therefore, LiaH could act as a peripheral membrane protein that senses SCE stress or changes in the membrane stability upon TatAy and TatCy overexpression. Importantly, the interaction of TatCy with LiaH was clearly detectable in a TatAy-deficient background, strengthening the view that not only TatAy, but also TatCy interacts directly with LiaH. Accordingly, the observed TatCy-LiaH interaction seems to suggest that TatCy overexpression may also lead to SCE stress.

Our results show that the P_{liaI} promoter is highly responsive to elevated TatAyCy levels in the cell. On the other hand, deletion of the *tatAyCy* genes had no detectable effect on the cellular LiaH levels. The latter can be explained by the fact that, in wild-type *B. subtilis* 168, the levels of TatAy and TatCy expression are extremely low [9,21,49]. However, this lack of effect of the *tatAyCy* deletion does not necessarily mean that the interaction between TatAyCy and LiaH is absent in wild-type *B. subtilis* cells, and that the observed interaction with LiaH upon overproduction of TatAyCy does not exist when this protein translocase is present at physiological levels. In particular, the finding that elevated LiaH production, which was triggered with the LiaRS-inducing cue bacitracin, led to elevated levels of TatAy is indicative of a TatAy-stabilizing activity of LiaH under physiological conditions, especially since *tatAy* gene expression is not modulated by LiaRS or SigW [40,41,50]. Conversely, we have previously shown that the cellular levels of TatAy are decreased in absence of its partner protein TatCy [19], which implies that TatAy requires a cognate partner protein for stabilization. In agreement with the idea that the interaction between TatAyCy and LiaH is specific, we observed that neither overexpression of TatAc, nor overexpression of the Tat substrate EfeB triggered substantially enhanced levels of LiaH. The fact that overexpression of TatAc did not elicit enhanced LiaH levels is particularly noteworthy, as it is in stark contrast with the massive LiaH induction observed upon TatAyCy, TatAy or TatCy overproduction [27]. In fact, this is in line with our previous observation that TatAc cannot functionally replace TatAy in Tat-dependent protein translocation [27], and it suggests that the LiaH response is associated with Tat function.

Interestingly, LiaH seems not only involved in responding to the cellular TatAyCy levels, but it also influences the activity of the TatAyCy translocase. Even though LiaH is not essential for the Tat-dependent protein translocation process per se, absence of LiaH affects the translocation of EfeB and leads to aberrant secretion of QcrA. A LiaH deficiency thus impacts on export of the two major cargo proteins of the TatAyCy translocase. These observations are reminiscent of a previous study, which showed that the absence of PspA may lead to exacerbated saturation of the *E. coli* Tat translocase for native and heterologous Tat cargo proteins [64]. By contrast, the absence of LiaH did not affect high-level protein secretion of the *B. subtilis* α-amylase AmyE via the Sec pathway, which indicates that, at least under the present experimental conditions, LiaH had a specific function in modulating the activity of the TatAyCy translocase. This conclusion is consistent with the results from other studies, which have implicated PspA and VIPP1 in Tat-dependent protein transport in *E. coli* [32] and thylakoids from *Pisum sativum* [65], respectively.

Altogether, our present study shows that LiaH is super-responsive to TatAyCy expression, and that a LiaH-deficiency has important consequences for TatAyCy-dependent protein export in *B. subtilis*. We propose that this relates to direct interactions between TatAyCy and LiaH. However, we can presently not fully exclude the possibility that the observed TatAyCy-LiaH interactions are a consequence of enhanced expression of the TatAyCy translocase in our experimental setup, and a corresponding response of the LiaRS system. In a previous study in *E. coli*, Alcock and coworkers observed that high expression levels of the

TatA, TatB and TatC components can lead to changes in the stoichiometry and behavior of the TatABC translocase [66]. Therefore, it is possible that TatAyCy overexpression could also lead to similar structural rearrangements in this translocase with consequences for membrane integrity that provoke a LiaRS response. Nevertheless, it is tempting to hypothesize that the molecular association of LiaH and TatAyCy relates to the recently reported membrane-weakening by TatA proteins, which suggests that membrane-weakening is fundamental to Tat-mediated protein translocation [46]. In turn, this would explain why cell envelope adaptation mediated by the LiaRS two-component system and minimal Tat translocation are functionally intertwined in *Bacillus*.

4. Materials and methods

4.1. Growth conditions

B. subtilis and *E. coli* strains were grown in Lysogeny Broth (LB) broth at 37 °C with shaking at 250 rpm. For transformation, *B. subtilis* was grown in Paris Medium (PM) as previously described [19]. When required the medium was supplemented with antibiotics: kanamycin (20 μg/ml), erythromycin (2 μg/ml), chloramphenicol (10 μg/ml), or bacitracin (30 μg/ml). *Lactococcus lactis* PAO1001 was grown at 30 °C without shaking in M17 broth supplemented with 0.5% glucose, or on M17 agar supplemented with 0.5% glucose (*w/v*) and erythromycin (5 μg/ml) for plasmid selection.

4.2. Plasmids and strains

Plasmids and strains used for this study are listed in Tables 1 and 2, respectively. Primers used to construct particular plasmids are listed in Table S2.

To overexpress TatAy and/or TatCy in *B. subtilis*, the pNZ8910 plasmid was amplified by PCR with primers containing overlapping regions for the *tatAy* and/or *tatCy* genes. The *tatAy* and/or *tatCy* genes were amplified with primers containing overlapping sequences for pNZ8910. The reverse primers also encoded a His_6 tag. All fragments were amplified with Phusion High Fidelity Polymerase and joined using the NEB Gibson Assembly® according to the manufacturer's instructions. The resulting plasmids were first introduced into L. *lactis* PAO1001 by electrotransformation. All constructs thus obtained were verified by PCR and sequencing, prior to their introduction in *B. subtilis*.

To overexpress TatAc in *E. coli* for subsequent purification and polyclonal antibody generation, the full-length *tatAc* gene was amplified from chromosomal DNA of *B. subtilis* 168 by PCR and cloned in pET26b. Likewise, for overexpression of TatAc in *B. subtilis*, the *tatAc* gene was amplified with specific primers and cloned in pNZ8910. Correct insertion of TatAc in pET26b and pNZ8910 was verified by PCR and sequencing.

4.3. Markerless gene deletion of liaH

The strain TMB1778 containing a markerless gene deletion of *liaH* was generated using the chromosomal integration-excision vector pMAD [67]. In brief, 1 kb fragments up- and downstream of *liaH* gene were amplified by using the primer pairs liaHclean_upfwd_BamHI/ liaHclean_uprev and liaHclean_downfwd/ liaHclean_downrev_*Sal*I, respectively (Table S2). Then, fragments were fused in a second joining PCR. The joining product and the chromosomal integration-excision vector pMAD were restricted with *Bam*HI and *Sal*I, and ligated resulting in plasmid pMAD-*liaH*clean. After sequencing the plasmid, *B. subtilis* 168 was transformed and cells were plated onto LB agar containing erythromycin (1 μg/ml), lincomycin (25 μg/ml) and X-Gal (100 μg/ml). Blue colonies with pMAD integrated in the *liaH* locus were picked, excision of the integrated pMAD from the chromosome was triggered by incubation at 42 °C, and white offspring colonies that had lost *liaH* along with the excised pMAD plasmid were identified by colony PCR.

4.4. Overexpression of TatAyCy, TatAy and TatCy

TatAyCy, TatAy or TatCy were overexpressed using the subtilin-inducible SURE system as previously described [42]. To this end, *B. subtilis* 168 containing pNZ-t*atAyCyHis* or pNZ-*tatCyHis* was plated on LB at 37 °C. Single colonies were used to inoculate 20 ml overnight cultures in LB with proper antibiotics, which were incubated at 37 °C with vigorous shaking (250 rpm). The following morning, the cultures were diluted to an OD_{600} of 0.05–0.08. Once an OD_{600} of 0.8 was reached, the overexpression of TatAyCy or TatCy was induced by addition of 1% subtilin. After 2.5 h incubation, cells were harvested by centrifugation (4 °C, 5500 × *g*, 10 min). To overexpress TatAy, we applied the genome-reduced strain IIG-Bs27-47-24 containing plasmid pNZ-*taAyHis* and followed the above procedure for overexpression of TatAyCy and TatCy.

4.5. Cell fractionation

Cells overexpressing TatAyCy, TatCy or TatAy were fractionated as previously described [30,68]. Briefly, the pellet was resuspended in protoplast buffer (0.1 M Tris-HCl pH 8.2 with 1 mg/ml lysozyme, 0.01% DNAse, 20 mM MgCl2, 20% sucrose, and one tablet of cOmplete Mini EDTA-free protease inhibitor cocktail™; Roche). The resulting protoplasts were collected by centrifugation (4000 xg, 10 min) and disrupted with a Precellys24 bead beater (Bertin Technologies, Montigny-le-Bretonneux, France). Subsequently, the cytoplasmic membrane fraction was obtained by ultracentrifugation (250,000 xg, 60 min), resuspended in solubilisation buffer (20 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, 0.1% DDM and solubilized overnight at 4 °C.

Table 1
Plasmids used in this study.

Plasmids	Description	Reference
pNZ8910	SURE expression vector; Em^R	[42]
pNZ-*tatAyCyHis*	pNZ8910 carrying the *tatAyCy* genes; only *tatCy* contains a 3' His_6 tag-encoding sequence; Em^R	[30]
pNZ-*tatAyCyStrep*	pNZ8910 carrying the *tatAyCy* genes; only *tatCy* contains a 3' StrepII tag-encoding sequence; Em^R	[30]
pNZ-*tatAyHis*	pNZ8910 carrying the *tatAy* gene with a 3' His_6 tag-encoding sequence; Em^R	This study
pNZ-*tatAc*	pNZ8910 carrying the *tatAc* gene; Em^R	This study
pNZ-*tatCyHis*	pNZ8910 carrying the *tatCy* gene with a 3' His_6 tag-encoding sequence; Em^R	This study
pNZ-*efeBstrepII*	pNZ8910 carrying the *efeB* gene with a 3' StrepII tag-encoding sequence, Em^R	This study
pBS3Clux *PliaI*	*sacA*::pCHIlux101; Cm^R	[70]
pGDL48	Constitutive expression vector; Ap^R, Km^R	[75]
pC*AyCy*	pGDL48 derivative; expresses the *tatAyCy* operon from a constitutive promoter; Ap^R, Km^R	[21]
pMAD	Integration-excision vector for markerless chromosomal gene deletions; ori pE194-Ts; MCS-P*clpB bgaB*; ori pBR322; Em^R in *Bacillus*; Ap^R in *E. coli*	[67]
pMAD-*liaH*clean	pMAD carrying the merged upstream and downstream *liaH* sequences	This Study
pET26b-*tatAc*	pET26b carrying the *TatAc* gene, Km^R	This study

Table 2
Strains used in this study.

Strain	Characteristics	Reference
Bacillus subtilis		
ATCC6633	Subtilin producer	[76]
168	*trpC2*	[77]
NZ8900	168 derivative, *trpC2*, *amyE*::*spaRK*, subtilin-inducible expression, Km^R	[42]
NZ8900 pNZ-*tatAyCyHis*	TatAyCy-His overexpression, TatCy contains a C-terminal His_6 tag, Km^R, Em^R	[30]
NZ8900 pNZ-*tatAyCyStrep*	TatAyCy-Strep overexpression, TatCy contains a C-terminal StrepII tag, Km^R, Em^R	[30]
NZ8900 pNZ-*tatCyHis*	TatCy-His overexpression, TatCy contains a C-terminal His_6 tag, Km^R, Em^R	This study
NZ8900 *tatAyCy*::*spec* pNZ-*tatCyHis*	TatCy-His overexpression in a TatAyCy-deficient background. TatCy contains a C-terminal His6 tag, Km^R, Em^R, Spc^R	This study
NZ8900 pNZ-*tatAc*	TatAc overexpression, Km^R, Em^R	This study
IIG-Bs27-47-24	Genome-reduced strain; Tat-related genes that are still present: *tatAd*, *tatCd*, *tatAy*, *tatCy*, *qcrA*, *efeB*, *ykuE*, *phoD*; Absent: *tatAc*	[78]
IIG-Bs27-47-24 *amyE*::*spaRK*	IIGBs-27-47-24 derivative carrying the *spaRK* genes in the *amyE* locus, Km^R	[79,80]
IIG-Bs27–47-24 *amyE*::*spaRK* pNZ-*tatAyHis*	TatAy-His overexpression, TatAy contains a C-terminal His_6 tag, Km^R, Em^R	This study
NZ8900 *sacA*::pCHIlux101	P_{liaI} *lux* reporter strain, Cm^R	This study
NZ8900 *sacA*::pCHIlux101 pNZ-*tatAyCyHis*	P_{liaI} *lux* reporter, *amyE*::*spaRK*, pNZ-*tatAyCyHis*, Cm^R, Km^R, Em^R	This study
168 X-*efeB*	*amyE*::*xylA-efeB(ywbN)-myc*	[21]
168 pC*AyCy*	Constitutive overexpression of TatAyCy	[20,21]
168 *X-efeB* pC*AyCy*	Xylose-inducible expression of *efeB-myc* and constitutive overexpression of TatAyCy, Km^R	This study
168 Δ*liaH* (TMB 1778)	*liaH* markerless deletion	This study
168 Δ*liaH X-efeB*	*liaH* markerless deletion and xylose-inducible expression of *efeB-myc*, Cm^R	This study
168 Δ*liaH X-efeB* pC*AyCy*	*liaH* markerless deletion, xylose-inducible expression of *efeB-myc* and constitutive overexpression of TatAyCy, Cm^R, Km^R	This study
CB15-14 AmyE	*degUHy32, amyE*::*xylR* P_{xylA} *comK-ermC, aprE*::P_{aprE}-*amyE cat*^{R} *Tbpn'*, Δ*upp*::*neo*^{R}, Em^R, Cm^R, Neo^R	[72]
CB15-14 AmyE Δ*liaH*	See CB15–14 AmyE, Δ*liaH*::*upp-phleo*^{R}-*cI* Em^R, Cm^R, Neo^S, $Phleo^R$	This study
NZ89100 pNZ-*efeBstrepII*	EfeB overexpression, EfeB contains a C-terminal strepII tag, Km^R, Em^R	This study
Lactococcus lactis		
PA1001	MG1363 derivative, *pepN*::*nisRK*, nisin-inducible expression, Δ*acmA* Δ*htrA*	[81]
Escherichia coli		
E. coli BL21(DE3)	*E. coli* host strain for protein overexpression	[82,83]
E. coli BL21(DE3) pET26b-*tatAc*	TatAc overexpression, Km^R	This study

Cm, chloramphenicol; Em, erythromycin; Km, kanamycin, Neo, neomycin; Phleo, phleomycin.

4.6. Metal affinity chromatography and size exclusion chromatography of TatAyCy-His

Purification of TatAyCy-His by metal affinity chromatography and size exclusion chromatography was performed using an ÄKTA Avant (GE Healthcare Life Sciences) as previously described with minor modifications [30]. A His-trap HP 1 ml column (Amersham Biosciences) was equilibrated with 3 column volumes at a flow rate of 1 ml/min with Buffer A (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM Imidazole, and 0.02% [*w/v*] DDM). Next, the solubilized membranes containing overexpressed TatAyCy-His were diluted to 10 ml in Buffer A and loaded onto the column at a flow rate of 1 ml/min. The column was washed 5 times with buffer A (flow rate 1 ml/min) and then TatAyCy-His along with associated proteins was eluted using a gradient of 5–300 mM imidazole in buffer B (flow rate 1 ml/min; 20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 0.02% (*w/v*) DDM). Elution fractions of 1 ml were collected and stored at 4 °C. Size exclusion chromatography was performed in SEC buffer (20 mM Tris, pH 8.9, 200 mM NaCl, 0.02 [w/v] DDM) using a Superdex 200 10/300 column (GE Healthcare Life Sciences). The chromatography experiments were repeated twice.

4.7. TatAc purification for antibody production

To obtain high levels of recombinant TatAc protein, *E. coli* DL21(DE3) carrying pET26b-*tatAc* was used to inoculate 5 L of LB broth. TatAc expression was induced with 0.4 mM IPTG when the culture reached an OD_{600} of ~1.0. After 2 h of growth in the presence of IPTG, cells were harvested by centrifugation. The collected pellets were resuspended in 25 ml of Buffer A, containing 20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM Imidazole, and supplemented with the cOmplete Mini protease inhibitor cocktail™ (Roche). Subsequently, the cells were disrupted by sonication with a Misonix Sonicator 4000–010 (3 min with 3 s pulses at pulse amplitude 50, followed by 3 min with 1 s pulse at amplitude 40), followed by two cycles of bead-beating (30 s, 3779 *g*) with a Precellys 24 bead beater. The cell lysate was then centrifuged (40 min, 10,000 rpm, 4 °C), and the supernatant was collected and supplemented with a final concentration of 0.1% DDM and incubated for 15 min at 4 °C. Next, TatAc was purified by metal affinity chromatography using an ÄKTA Avant as described above. TatAc-containing elution fractions were desalted with a HiTrap Desalting column (50 mM Tris-HCl, pH 6.8, 400 mM NaCl) and concentrated using a 5 K MWCO Pierce™ Protein Concentrator. The purified TatAc protein was used for rabbit immunization according to a standard protocol (Eurogentec).

4.8. LDS-PAGE and Western blotting

Proteins were separated on 10% pre-cast Bis-Tris NuPaGE gels, which were either stained with SimplyBlue SafeStain (Thermo Fisher) or used for Western blotting where the separated proteins were transferred to a nitrocellulose membrane (Amersham™ Protran® 0.45 µm, GE Health Care Sciences) by semi-dry blotting. Membranes were blocked overnight with 5% (*w/v*) skim milk. The next day, the membranes were washed three times for 5 min with phosphate-buffered saline plus Tween20 (PBS-T). The membranes were then incubated with polyclonal rabbit antibodies (1:5000) raised against EfeB, LiaH, QcrA, TatAc, TatAy or TrxA, or monoclonal antibodies (1:5000) specific for the His_6 tag (Invitrogen)(Table 3). After 1 h incubation, the membranes were washed with PBS-T, and incubated for 45 min with fluorescent goat-anti rabbit IgG® 680RD (1:5000) or goat-anti mouse IgG® 680RD (1:5000; LI-COR). The membranes were washed three times for 5 min with PBS-T, and twice for 5 min with PBS. Lastly, bound antibodies were visualized with an Odyssey Infrared Imaging System (LI-COR). All Western blots were performed in duplicate or triplicate.

4.9. GeLC−MS analysis

Samples were subjected to GeLC-MS/MS analysis as previously described [69]. Briefly, upon staining with SimplyBlue SafeStain, gels

Table 3
Antibodies used in this study.

Antibody	Characteristics	Reference
His_6 tag Monoclonal Antibody	Mouse monoclonal antibody	ThermoScientific MA1–21315
Anti-LiaH	Rabbit polyclonal antibody	[44]
Anti-EfeB	Rabbit polyclonal antibody	[20]
Anti-TatAy	Rabbit polyclonal antibody; note that this antibody recognizes both TatAy and His_6 tags	[26]
Anti-TatAc	Rabbit polyclonal antibody	This study
Anti-TrxA	Rabbit polyclonal antibody	[84]
Anti-QcrA	Rabbit polyclonal antibody	[18,55]
StrepMAB classic	Mouse monoclonal antibody	iba StrepMAB classic 2–1507-001

were washed twice with water to remove excessive stain. Protein bands of interest were excised, transferred into low-binding Eppendorf tubes, and washed/destained at least three times for 15 min with 750 µL of gel washing buffer (0.2 M ammonium bicarbonate in 30% [*v*/v] acetonitrile) at 37 °C under vigorous shaking. The destained gel pieces were dried in a vacuum centrifuge at 30 °C and rehydrated with trypsin solution (2 µg of modified trypsin [Promega] in 1 mL of water) for 15 min. Excess trypsin solution was removed, and digestion was performed overnight at 37 °C. Next day, the gel pieces were covered with water, and peptides were eluted from the gel matrix by immersion of the reaction tube in an ultrasonic bath for 15 min. The supernatant containing the peptides was transferred to a glass vial and concentrated to a final volume of 10 µL in a vacuum centrifuge. For LC − MS/MS analyses of 1D gel samples, in-house self-packed columns were prepared and used with an EASY-nLC II system (Thermo). In brief, fused-silica emitter tips with an inner diameter of 100 µm and an outer diameter of 360 µm were prepared by using a P-2000 laser puller (Sutter Instruments). The resulting emitter tips were then packed with Aeris C18 reversed-phase material (3.6 µm particles) in a custom-built pressure bomb to obtain a 20 cm nano-LC column. The peptides were loaded onto the column by the LC system with 10 µL of buffer A (0.1% [v/v] acetic acid) at a constant flow rate of 500 nL/min without trapping. The peptides were subsequently eluted using a nonlinear 100 min gradient from 1 to 99% buffer B (0.1% [v/v] acetic acid in acetonitrile) with a constant flow rate of 300 nL/min and injected online into the mass spectrometer. MS and MS/MS data were acquired with a Linear Trap Quadrupole Orbitrap (Thermo). After a survey scan at a resolution of 30.000 in the Orbitrap with activated lockmass correction, the five most abundant precursor ions were selected for fragmentation. Singly charged ions as well as ions without detected charge states were not selected for MS/MS analysis. Collision-induced dissociation (CID) fragmentation was performed for 30 ms with normalized collision energy of 35, and the fragment ions were recorded in the linear ion trap.

Database searching was done with Sorcerer-SEQUEST 4 (SageN). After extraction from the raw files, *.dta files were searched with Sequest against a target−decoy database with a set of common laboratory contaminants. The target database was the Uniprot reference database of *B. subtilis* 168 (downloaded May 21, 2014). The resulting *.out files were compiled with Scaffold 4. Proteins were only considered as identified if at least two unique peptides matching solid quality criteria (delta cN > 0.1 and XCorr > 2.2; 3.3; 3.75 for doubly, triply, or higher charged peptides) had been assigned, resulting in a false-positive rate (FPR) below 0.1% on protein level.

4.10. Co-Immunoprecipitation

For co-IP analyses to assess interactions between TatAyCy, TatCy or TatAy with LiaH, strains were grown in LB and induced with 1% subtilin when an $0D_{600}$ ~0.8 was reached. Isolation of cytoplasmic membranes was performed as described above. Next, 50 µl of Protein A Dynabeads (Thermo Scientific) were washed with PBS-T, and incubated with 50 µl of anti-His_6 antibody and 150 µl of PBS-T for 30 min. The beads were subsequently washed with 200 µl of PBS-T and incubated with 150 µl of solubilized cytoplasmic membrane fractions mixed with 150 µl of PBS-T for 1 h under rotation. The beads were then washed three times with 800 µl of PBS-T, and eluted in 40 µl of 2× LDS loading dye at 80 °C for 10 min. Upon elution, the samples were subjected to LDS-PAGE and Western blotting. All co-IP experiments were performed in triplicate.

4.11. Luminescence assays

Luminescence assays were performed in triplicate as previously described with minor modifications [70]. Single colonies were used to inoculate 10 ml of LB medium supplemented with antibiotics. The cultures were incubated overnight at 37 °C (250 rpm). The following morning, each culture was diluted 500-fold in 10 ml of LB medium without antibiotics and incubation was continued until an OD_{600} of 0.2–0.5 was reached. The cultures were then diluted in fresh LB to an OD_{600} of 0.015, and 150 µl aliquots of each culture were transferred into a Nunc™ MicroWell™ 96-Well Optical-Bottom Plate (Thermo Scientific). The OD_{600} and relative light units (RLU) were measured for 16 h using a Synergy™ plate reader (BioTek). In *B. subtilis* carrying pNZ-*tatAyCyHis*, the overexpression of TatAyCy-His was induced with 1% subtilin when an OD_{600} of 0.1 was reached. Likewise, a LiaRS-mediated cell envelope stress response was induced with 30 µg/ml bacitracin at an OD_{600} of 0.1. To calculate the luminescence the RLU was divided by the OD_{600}.

4.12. Assessment of EfeB and QcrA* secretion

Bacteria were grown overnight in LB medium with appropriate antibiotics. Next morning, the bacteria were diluted 50-fold in fresh LB medium and growth was continued for 3 h. To induce the X-*efeB* cassette (formerly referred to as X-*ywbN*), 1% xylose was added to the medium. Cells were then separated from the growth medium by centrifugation (17,000 xg, 10 min). Proteins in the growth medium fraction were precipitated with 10% trichloroacetic acid (TCA) as previously described [71]. The cell pellet was resuspended in LDS loading buffer and disrupted with 0.1 µm glass beads by bead-beating (three cycles 30 s, 3779 *g*, 30 s intervals) using a Precellys24 bead beater. The cytoplasmic and extracytoplasmic proteins were separated on 10% precast Bis-Tris NuPaGE gels, and the presence of EfeB, LiaH, QcrA, TatAy or TrxA was visualized by Western blotting as described above.

4.13. Assessment of high-level AmyE secretion in the absence of LiaH

Deletion of the *liaH* gene from the *B. subtilis* strain CB15–14 Δ*upp*::*neo*^{R} was performed as described previously [72], using the modified mutation delivery method described by Fabret et al [73]. The 5′ and 3′ regions of *liaH* were amplified using the primer pairs designated LiaH·P1/LiaH·P2 and LiaH·P3/LiaH·P4 (Table S2). The resulting fragments were fused to a deletion-cassette containing a phleomycin resistance marker, the *upp* gene and the *cI* gene. The fusion product was then used to transform *B. subtilis* CB15–14 Δ*upp*::*neo*^{R}. The deletion of *liaH* was confirmed by PCR using the primer combinations LiaH·P0/LiaH·P4 and LiaH·P0/CI2.rev (Table S2). The chromosomal AmyE expression cassette was introduced by transformation and, subsequently, amplified by growing transformants at increasing chloramphenicol concentrations up to 25 µg/ml [72]. Sec-dependent secretion of AmyE was assayed as described previously [72,74]. Bacteria were grown for ~8 h in LB broth with 25 µg/ml chloramphenicol. The cultures were then diluted 1000-fold in MBU medium [72] with 2.5 µg/ml chloramphenicol and incubated for approximately 16 h at 37 °C under vigorous shaking (250 rpm). After measuring and correcting for the optical density at 600 nm (OD_{600}), equal amounts of cells were separated from

the growth medium by centrifugation. Proteins in the cellular and growth medium fractions thus obtained were analyzed by LDS-PAGE as described above and the gels were subsequently stained with SimplyBlue™ SafeStain (Life Technologies).

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Author contributions

M.B·C., M.M. and J.M.v.D conceived and designed the experiments.

M.B.C., M.M., M.A.V., R.A.S, L.S., J.N., and D.W. performed experiments and analyzed the data.

J.N., D.B., and D.W. contributed strains and reagents.

G.G., A.O., D.B. and J.M.v.D. supervised the project.

M.B.C. and J.M.v.D. wrote the manuscript.

All authors have read and approved the manuscript

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The mass spectrometry data are deposited in the ProteomeXchange repository PRIDE (https://www.ebi.ac.uk/pride/) with the dataset identifier PXD012842.

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